Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/003910

International filing date: 07 February 2005 (07.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/542,499
Filing date: 06 February 2004 (06.02.2004)

Date of receipt at the International Bureau: 27 June 2005 (27.06.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

1334001

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

June 15, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/542,499*
FILING DATE: *February 06, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/03910*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Attorney Docket No. 00952-01

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Certificate Under 37 CFR 1.10
Date of Deposit: February 6, 2004
I hereby certify that this correspondence is being deposited with the United States Postal Service as "Express Mail" service under 37 CFR 1.10 on the date indicated above addressed to Mail Stop Provisional Application, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450

Sue Ann Carr
Express Mail No. ER 861 427 885 US

Address to: Mail Stop Provisional Application
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450
(703) 308-4357

This is a request for filing a Provisional Application for Patent under 37 CFR 1.53(c)

Inventor(s) and Residence(s) (city and either state or foreign country):

Last Name	First Name	Middle Initial	City	State or Country
Gilmer	Linda		Charlottesville	Virginia
Mandal	Arabinda		Charlottesville	Virginia
Wolkowicz	Michael	J.	Charlottesville	Virginia
Klotz	Kenneth	L.	Esmont	Virginia
Herr	John	C.	Charlottesville	Virginia

Title: **COMPOSITION FOR IDENTIFYING SPERM FOR FORENSIC APPLICATIONS**

29 Sheets of specification.
2 Sheets of drawings.

University of Virginia Patent Foundation claims small entity status as a nonprofit organization (37 CFR §§1.27(a)(3) and (c)). The Commissioner is hereby authorized to charge the Small Entity Fee of $80 to Deposit Account No. 50-0423.

Please direct all communication relating to this application to:
John P. Breen, Esq.
Patent Counsel
University of Virginia Patent Foundation
1224 West Main Street, Suite 1-110
Charlottesville, VA 22903 U.S.A.

Customer No. 34444
Telephone: (434) 243-6103
Fax: (434) 924-2493

This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The government has certain rights in the invention.

YES ☒ NO ☐ Grant No. NIH T32 HD07382, DK07642 and U5429099

Dated: February 6, 2004

Respectfully submitted,

By: _____
John P. Breen (Reg. No. 38,833)

Composition for Identifying Sperm for Forensic Applications

US Government Rights

This invention was made with United States Government support under Grant Nos. T32 HD07382, T32 DK07642, and U54 29099, awarded by National Institutes of Health, National Institute of Justice No. 2000-IJ-CX-K013 and Federal Bureau of Investigations No. 115744. The United States Government has certain rights in the invention.

Background

Sexual assault evidence recovered from a victim is an admixture of various cell types and fluids from both victim and assailant. In cases of vaginal assault, cells originating from the victim include cervical and vaginal epithelial cells, erythrocytes (red blood cells), white blood cells, various vaginal flora, including species of *Lactobacillus*, *Candida*, *E. coli*, as well as cervical mucus and minor contributions from uterine "milk". Semen, the male component, contains roughly 85% seminal fluid originating from prostate and seminal vesicles, epithelial cells from these organs, spermatozoa, and epididymal fluid (15% of the ejaculate volume) and may contain white blood cells and various bacterial, viral or fungal commensals. In various cases of oral assault, buccal epithelial cells and buccal flora are often present as part of the female component. In cases of anal assault, a variety of intestinal and colonic epithelial cells, secretions, foodstuffs, and bacteria may be present in the victim's component.

Currently, the only stains available to aid in the identification of sperm are nuclear and cytoplasmic stains [such as the Christmas Tree stain] which are not specific for sperm but stain a variety of cells including vaginal and cervical epithelial cells, bacteria and cells sloughed from the male accessory sex glands. This leaves the positive identification of sperm relying on discovery of the characteristic shape and form of intact sperm, which may prove difficult as the sperm head and tail separate very easily after the sperm is dried and eluted from swabs. This problem of positive identification is particularly problematic where few numbers of sperm are present in the midst of a large number of other cells and debris. In such instances it may take a very long time for the forensic scientist to scan microscope slides in order to positively confirm the presence of sperm.

Accordingly, there is a need for a highly specific sperm stain that will make the sperm stand out in a field of debris and other cell types. Furthermore, since the sperm head and tail may become separated during acquisition, storage or handling of a forensic sample, (and one or the other subsequently lost) the use of sperm head or tail specific target proteins is also desired so each may be separately positively identified. In accordance with one embodiment one or more sperm-specific protein markers located on the head and/or the tail are utilized to allow the rapid detection of sperm in smears from forensic samples.

Once sperm are isolated from other components present in a forensic sample, PCR based analysis of sperm DNA can identify the source individual with a high degree of certainty. Many criminals have been identified after comparison of their DNA to the patterns recorded in the convicted offenders database (CODIS). Because of the great sensitivity of the PCR method it is possible to obtain useful data from a small number of recovered sperm, even as few a single sperm. However, with the advent of PCR based reactions and their increased sensitivity, the problem of defining the cellular source of amplified DNA and assigning, beyond a reasonable doubt, that source to the assailant, has proved a more difficult undertaking. A need for purer input DNA is thus inherent in the PCR protocol where all DNAs, including contaminating species, undergo amplification prior to analysis.

One approach for identifying and isolating human sperm in a forensic sample comprises the use of ligands that specifically bind to unique sperm surface compounds. Such sperm specific compounds should be readily accessible for binding to a ligand (e.g. an antibody) and yet the sperm specific compound must be sufficiently stable that the compound is still present on the sperm, and capable of being recognized by the ligand, after recovery and storage of a forensic sample. As described herein applicants have discovered that sperm membrane antigens are often lost and are absent from sperm recovered from dried swabs prepared in sexual assault cases. This is due to the fact that the plasma membrane is absent in many sperm eluted from post-coital swabs. Accordingly, the present invention is directed to a method of identifying and isolating sperm from a forensic sample. The method utilizes reagents that specifically bind to sperm specific compounds that are stable and persist on sperm heads and/or tails during the time and procedures used to recover forensic samples.

Summary of Various Embodiments of the Invention

The present invention is directed to compositions and methods for identifying and isolating sperm and/or sperm DNA from forensic samples. In one embodiment the isolated sperm DNA will be used for forensic DNA analysis of the "male component" in sexual assault evidence. The recovered sperm DNA will be subjected to polymerase chain reaction (PCR) analysis of short tandem repeat loci providing an enabling technology to assist the development of the National Convicted Offender Database (CODIS).

Brief Description of the Drawings

Fig. 1 demonstrates that an antibody against CABYR-A can be used to identify sperm tails in a post-coital sample eluted from cotton swab.

Fig. 2 demonstrates that an antibody against ESP can be used to identify sperm heads in a post-coital sample eluted from cotton swab.

Detailed Description of Embodiments

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

As used herein, the term "linkage" refers to the connection between two groups. The connection can be either covalent or non-covalent, including but not limited to ionic bonds, hydrogen bonding and hydrophobic/hydrophilic interactions.

As used herein, the term "secondary antibody" refers to an antibody that binds to the constant region of another antibody (the primary antibody).

A "detectable marker" is an atom or molecule that permits the specific detection of a molecule comprising the marker in the presence of similar molecules without a marker. Markers include, for example radioactive isotopes, antigenic determinants, enzymes, nucleic acids available for hybridization, chromophors, fluorophors, chemiluminescent molecules, electrochemically detectable molecules and molecules that provide for altered fluorescence-polarization or altered light-scattering

As used herein the term "solid support" relates to a solvent insoluble substrate that is capable of forming linkages (preferably covalent bonds) with various compounds. The support can be either biological in nature, such as, without limitation, a cell or bacteriophage particle, or synthetic, such as, without limitation, an acrylamide derivative, agarose, cellulose, nylon, silica, or magnetized particles.

As used herein the term "magnetic particles" refers to particles that are responsive to a magnetic field.

As used herein, the term "antibody" refers to a polyclonal or monoclonal antibody or a binding fragment thereof such as Fab, F(ab')2 and Fv fragments.

Embodiments

The present invention is directed to compositions and methodologies directed to rapidly identifying human sperm in sexual assault evidence. In one aspect of the invention, protocols are designed for rapidly determining the presence of sperm in a sample when numbers of sperm are low, for example in a sample eluted from sexual assault swabs. Reduction in the amount of time required to positively identify human sperm in sexual assault samples is anticipated to provide a cost savings in forensic practice as well as expedite the number of cases processed, particularly in cases where sperm are mixed with a variety of other cells and unknown material. In another aspect of the invention reagents are prepared for rapidly isolating and purifying sperm from sexual assault evidence to allow recovery and analysis of sperm DNA.

Currently, the only stains available to aid in the identification of sperm are nuclear and cytoplasmic stains which are not specific for sperm but stain a variety of cells including vaginal and cervical epithelial cells, bacteria and cells sloughed from the male accessory sex glands. This leaves the positive identification of sperm relying on the characteristic shape and form of intact sperm, which may prove difficult as the sperm head and tail separate very easily after the sperm is dried and eluted from swabs. This problem of positive identification is particularly problematic where low numbers of sperm are present in the midst of a large number of other cells and debris.

In accordance with one embodiment of the present invention, antibodies directed against sperm specific proteins are used to identify and isolate sperm cells from complex biological mixtures. For an antigen to be useful in sperm immunoselection it must be present and accessible on the sperm, and must not react with other cell types, including vaginal epithelial cells that may be present in the biological sample. The selection of the sperm specific protein is particularly important for samples that are recovered from dried swabs or forensic evidence, since the sperm plasma membrane is frequently lost during the recovery of such samples. In addition, it is also desirable that the selected sperm specific target proteins are restricted to the head or the tail so that in instances when the heads separate from the flagella, each can be positively identified.

Antibodies to sperm specific polypeptides or peptide fragments thereof may be generated using methods that are well known in the art. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with a sperm specific polypeptide or peptide fragment thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-

hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for epitopes of SLLP polypeptides together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to one embodiment of the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce protein-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for sperm surface proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). Antibodies generated in accordance with the present invention may include, but are not limited to, polyclonal, monoclonal, chimeric (i.e. "humanized" antibodies), single chain (recombinant), Fab fragments, and fragments produced by a Fab expression library.

Several sperm-specific proteins have been previously described [see US Patent No 5,436,157 (SP-10), PCT/US99/24973 (Span-X), PCT/US01/01715 (CBP86), PCT/US00/02675 (AKAP) and PCT/US01/01717 (ESP & SAMP32), the disclosures of which are incorporated herein], that have the potential to allow the rapid detection of sperm in smears from forensic samples. However, as reported in Example 2 of the present invention sperm specific proteins that are located on the plasma membrane may not be retained on sperm that have been subjected to standard forensic recovery procedures. Accordingly, an effective composition for identifying sperm in a complex biological mixture should be based on targets that persist in sperm and can be detected when swabs are collected and allowed to dry before analysis of the recovered sample. In accordance with one embodiment, the sperm specific targets will be selected from those that persist on sperm for extended time periods up to 72 hours after intercourse.

As reported herein, see Example 2, ESP, SPAN-X, CABYR, SP-10, and SAMP32 are present and can be detected in many sperm when swabs are collected one to two hours after intercourse. In this experiment the sperm were stained with a specific antibody followed by a fluorescently conjugated secondary antibody. In addition, a sperm specific tail protein, AKAP3, can be detected in sperm recovered from swabs and will stain with a very bright fluorescent signal over the principal piece of the tail. It is expected that the fibrous sheath proteins CABYR and AKAP3 will be the most resilient and will be detectable for the longest time period.

In accordance with one embodiment of the invention a composition for labeling sperm heads and/or sperm tails is provided. More particularly, a composition is provided that specifically binds to post-coital sperm cells. In one embodiment the composition comprises an antibody that binds to a polypeptide selected from the group consisting of SEQ ID NO: 1 (SP-10), SEQ ID NO: 2 (CABYR), SEQ ID NO: 3 (ESP), SEQ ID NO: 4 (SAMP32), SEQ ID NO: 5 (SPAN-X), and SEQ ID NO: 6 (AKAP). In one embodiment the antibody is a monoclonal antibody. In another embodiment the sperm labeling composition will comprise a cocktail of two or more antibodies. In a further embodiment the composition will comprise two or more sperm specific antibodies, each staining a different head or tail protein of the sperm; and more particularly the composition will contain at least one antibody that binds to sperm head specific protein and one antibody that binds to sperm tail specific protein. Alternatively,

the cocktail may contain an antibody that binds to a sperm specific protein that is located on both the head and the tail of the sperm and is retained at least 2 hours after ejaculation.

The antibodies of the present invention can be combined with a carrier or diluent to form a composition. In one embodiment, the carrier is a pharmaceutically acceptable carrier. In another embodiment the antibody is linked to a solid support. In another embodiment the antibody is linked to a detectable marker.

The protocols for identifying sperm cells using sperm specific antibodies can employ a variety of detectable markers that are either directly linked or indirectly linked to the sperm specific antibody. In one embodiment the antibody is labeled either directly or indirectly, using an immunofluorescence compound and techniques known to those skilled in the art. In the direct method the monoclonal antibodies are labeled directly with a fluorochrome. In the indirect method, the fluorochrome is attached to a secondary antibody that recognizes the sperm specific monoclonal antibody. The indirect method has the advantage that it can amplify the fluorescent signal by binding more fluorochrome at the antigen site, thus its potential fluorescent signal on sperm may be stronger than the direct method, especially at low antibody-conjugate concentrations. A drawback of this method is that it employs two separate steps of antibody addition. The direct method has the advantage that it reduces the number of washing steps and is quicker. The use of a single labeled immunoreagent also reduces the background fluorescence by eliminating non-specific binding of the secondary antibody. One possible drawback of using a single labeled immunoreagent is that at low antibody-antigen ratios the fluorescent signal may be lower than the indirect method. In one embodiment the sperm specific antibody is a monoclonal antibody that has been directly conjugated to a fluorochrome. Using fluorescence microscopy, the equatorial band signal for a positive head or a fluorescing sperm tail is very strong and easily identifiable at 400x, even if the head and tail have separated.

In accordance with one embodiment a composition for labeling sperm cells comprises an antibody specific for the equatorial segment protein (ESP) protein (SEQ ID NO: 3) and an antibody that is specific for a protein selected from the group consisting of AKAP3 (SEQ ID NO: 6) and CABYR (SEQ ID NO: 2). The ESP protein represents an epitope in the sperm head whereas the AKAP3 and CABYR proteins represent epitopes in the sperm tail. In one embodiment the antibodies used are monoclonal antibodies.

The 3A4 monoclonal antibody binds to the equatorial segment protein (ESP) and stains the principal segment of the flagellum. The 3C6 monoclonal antibody binds to the calcium binding tyrosine phosphorylated protein (CABYR-A). Both antibodies have been shown to stain sperm present in post-coital evidence which has been stored for up to two years. When used in immunofluorescent microscopy using FITC conjugated secondary antibodies, the sperm are easily identified as they fluoresce brightly against a negative background. The head staining monoclonal antibody 3C6 gives a characteristic band across the mid-region of the sperm head that corresponding to the domain of the equatorial segment. The 3A4 monoclonal antibody stains the principal segment of the flagellum most intensely.

The present invention also provides a method for detecting the presence of human sperm in a biological sample. The method comprises the steps of contacting a sample with a labeled antibody (wherein the antibody specifically binds to a human sperm specific protein that is retained and is accessible to an antibody after the sperm containing specimen has been dried and subsequently rehydrated) and detecting the presence of the labeled antibody. The sperm cell identification system of the present invention rapidly identifies the presence of sperm in a recovered sample, including sperm recovered from dried stains on clothing, from vaginal swabs, from material collected by lavage with physiological saline, or from any suspension which includes sperm cells. In one embodiment the method further comprises the step of removing unbound and non-specific bond material as a means of purifying one or more sperm cells from a sample. In one embodiment, the antibody used in the method of identifying and/or isolating sperm is an antibody that binds to a sperm protein selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6.

The protocol described herein using antibodies to label sperm cell components is projected to be especially useful when the head and tail have become separated, and the shape and form of the sperm under light microscopy are difficult to discern. Using fluorescent microscopy, the equatorial band signal for a positive head or a fluorescing sperm tail is very strong and easily identifiable, even if the head and tail have separated. In view of the fact that sperm membrane antigens are often lost and absent from sperm recovered from dried swabs from sexual assault cases, the present invention is directed to antibodies that are specific for sperm cell specific antigens that have been

shown through experimentation to be retained on sperm eluted from dried post-coital swabs. In one embodiment, the target antigens are selected from those that are retained on sperm eluted from dried post-coital swabs which have been stored for greater than 72 hours, and even up to two years.

A number of anti-sperm antibodies have been tested, using immunofluorescent staining, to confirm the antibodies would react specifically with sperm and not epithelial cells present in samples recovered from one hour post-coital swabs. Five different antibodies directed against SP-10, ESP, SPAN-X, SAMP32, and CABYR passed this initial screen.

SP-10

Background on discovery and cloning.

The testis/sperm-specific, intra-acrosomal sperm antigen SP-10 was identified using MHS-10, a mAb generated against whole human spermatozoa. SP-10 was identified by immunblot analysis as a series of protein bands (18-34 kDa), the polymorphism of which was attributed to alternative splicing and endoproteolytic cleavage. Ultrastructural and biochemical studies indicated that SP-10 is a hydrophobic protein localized to the luminal aspect on the inner and outer acrosomal membranes.

Forensic Results.

SP-10 appears to remain on the head of sperm recovered from one hour post-coital swabs. The immunofluorescent image is of a fluorescent cap shaped organelle. The MHS-10 mAb reacts strongly with.these sperm heads and not with epithelial cells from the samples.

ESP-Sperm Equatorial Segment Protein:

Background on discovery and cloning.

Two protein spots on a 2-D gel of 36 and 38 kDa and pI 5.1 that reacted with infertile male sera (autoantigenicity) and with ConA (indicating glycosylation) were microsequenced and cloned. The 1.4 Kb cDNA included three in-frame peptides microsequenced from the original spot and hybridized to a single 1.4 Kb testis-specific transcript on a multiple-tissue Northern blot and to testis and placental mRNA on a dot blot of 76 tissues. Computer analysis of the open reading frame (ORF) demonstrated 29% identity and 49% homology over a 68 amino acid C-terminal region (amino acids

278-343) to murine osteoglycin, a secreted extracellular matrix protein. Generation of monospecific rat immune sera allowed localization of the ESP protein to the equatorial segment of human sperm by immunofluorescent and electron microscopy.

Forensic Results:

Antibody to ESP reacted with the equatorial segment of the sperm head but not with epithelial cells in cells recovered from one hour post-coital swabs. Due to the frequency with which sperm heads are separated from tails in cells recovered from swabs, antibodies directed against proteins found in the sperm head are most useful for an immunoselection device.

SPAN-X-Sperm Protein Associated with the X Chromosome

Background on discovery and cloning.

SPAN-X is a structural protein associated with the nuclear envelope of spermatozoa. Immunofluorescent labeling demonstrates that SPAN-X is localized to nuclear craters and cytoplasmic droplets of ejaculated human and chimpanzee spermatozoa. Ultrastructurally, the SPAN-X protein is associated with membranous structures within nuclear vacuoles and with the redundant nuclear envelope of human spermatozoa. The ultrastructural localization of the insoluble SPAN-X protein suggests that SPAN-X is a structural component of the sperm nuclear envelope or is associated with structural components of the nucleus, possibly the nuclear matrix. SPAN-X is the first protein specifically localized to these poorly characterized structures of the mammalian sperm nucleus and the first example of a testis-specific protein localized to the nuclear envelope of spermatids.

Significantly, 50% of ejaculated human spermatozoa exhibited immunofluorescent labeling with the SPAN-X antisera. The localization of SPAN-X to 50% of spermatozoa and its X-linked expression by haploid spermatids initially suggested that SPAN-X might be associated with only X-bearing spermatozoa. However, dual labeling of spermatozoa utilizing FISH for the X or Y chromosome and indirect immunofluorescence for the SPAN-X protein demonstrates that SPAN-X is equally distributed between X- and Y-bearing spermatozoa suggesting that SPAN-X mRNA and/or protein is shared within spermatid cohorts in the testis via cytoplasmic bridges.

Forensic Results.

Although SPAN-X is present in only 50% of sperm, the monoclonal antibody A9 generated against recombinant SPAN-X protein would be a valuable component of an antibody mix for sperm immunoselection. A9 was found to react with sperm heads in post-coital samples recovered from swabs, while A9 did not react with epithelial cells. Furthermore, this antibody stains SPAN-X localized to the highly convoluted redundant nuclear envelope which lies just beneath the plasma membrane in the cytoplasmic droplet of the spermatozoa. The plasma membrane in this region is easily disrupted thus exposing the SPAN-X protein and allowing binding of the A9 mAb.

CABYR - Calcium Binding Tyrosine Phosphorylation Regulated Fibrous Sheath Protein Involved in Capacitation:

Background on discovery and cloning.

CABYR was identified as acidic (pI 4.0) 86 kDa isoforms of a novel, polymorphic, testis-specific protein that were tyrosine phosphorylated during *in vitro* capacitation and bound calcium[45] on 2-D gels. CABYR is the first demonstration of a sperm protein that gains calcium binding capacity when phosphorylated during capacitation. Recombinant CABYR has been produced and used to immunize rats to produce polyclonal antisera. Using these sera for immunofluorescent and immuno-electron microscopy we have determined that CABYR localizes to the principal piece of the human sperm flagellum in association with the fibrous sheath

Forensic Results.

By immunofluorescent staining on samples recovered from post-coital swabs, this probe definitively identified sperm tails. This testis specific gene product offers an excellent target to detect sperm tails.

SAMP32 - A Testis-specific, Isoantigenic Acrosomal Membrane-associated Protein:

Background on discovery and cloning.

SAMP32 was identified in 2-D gel Western blots of sperm extracts containing hydrophobic proteins that partitioned into Triton X-114. Four protein spots with pIs ranging from 4.5 to 5.5 and apparent molecular weights from 32 to 34 kDa were sequenced by mass spectrometry and found to contain common peptide sequences. Cloning the corresponding cDNA revealed that these protein spots were products of a single gene (SAMP32) encoding a protein of 32 kDa with a predicted pI of 4.57.

SAMP32 has a potential transmembrane domain in the carboxyl terminus and is phosphorylated *in vivo* on serine 256. Northern blotting of 8 human tissues and RNA dot blotting of 76 human tissues showed that SAMP32 expression was testis-specific. A recombinant form of SAMP32 was produced in *E. coli* and rat polyclonal sera were produced to this recombinant SAMP32. The antisera strongly stained the equatorial segment and faintly stained the acrosomal cap of ejaculated human spermatozoa by immunofluorescence. Immunoelectron microscopy showed that SAMP32 was associated with the inner acrosomal membrane in the principal and the equatorial segments of the sperm acrosome.

Forensic Results

The rat polyclonal to SAMP32 used here reacted strongly with sperm heads from the post-coital samples. Sperm are identified by a cap shaped pattern or a bar shaped pattern of immunofluorescence. A low level of reactivity was observed with the epithelial cells. This likely can be eliminated by using a higher dilution of the antibody or a monoclonal antibody when one is available.

In accordance with one embodiment, the present invention is directed to a method of purifying sperm DNA from a biological sample that comprises multiple cell types. The method comprises selecting male germ cells and separate them from other cell types using the described sperm cell selection system. The DNA can then be recovered from the isolated male germ cells and amplified by a PCR reaction using techniques known to those skilled in the art. In accordance with one embodiment, a sperm immunoselection method is used to isolate highly pure sperm DNA for subsequent amplification. In accordance with one embodiment of the present invention sperm heads are purified from a complex biological sample through the use of antibodies that target sperm specific antigens that are located internal to the sperm plasmid membrane. Suitable sperm specific antigens include SP-10, ESP, SPAN-X, SAMP32, AKAP3 and CABYR. Since the sperm cells recovered will differ in the extent of the loss of their membranes depending on the source and age of the sample as well as the procedure used to recover the sperm and storage conditions, in one preferred embodiment a cocktail of antibodies will be used. The sperm immunoselecting composition will comprise two or more antibodies that bind to unique human sperm epitopes located on different layers of the sperm cell membranes.

Advantageously, antibodies specific for human sperm antigens can be selected and combined such that the cocktail will immunoselect a broad range of sperm cells that vary in the degree in the amount of retained sperm membranes, but excluding those that are lost with the sperm plasma membrane. For example one such cocktail would include antibodies directed against the sperm specific proteins SP-10, ESP and SPAN-X. In an alternative embodiment the cocktail may comprise antibodies directed against the sperm specific proteins CABYR, SP-10, ESP and SPAN-X. In one embodiment a cocktail of anti-sperm antibodies against multiple antigens is provided wherein the cocktail comprises at least two, and more preferably three antibodies, wherein the antibodies are selected from the group consisting of the AKAP3 antibody, SP-10 antibody, ESP antibody, SAMP32 antibody, CABYR antibody and SPAN-X antibody. In one embodiment the cocktail further comprises an antibody directed against protamine 1 and protamine 2. In one preferred embodiment the antibodies are monoclonal antibodies.

The anti-sperm antibodies directed to human sperm surface antigens can be bound to solid supports (such as magnetic particles) to enhance cell separation methods and reduce the presence of contaminating cells in forensic evidence. The sperm specific antibodies can be bound to the solid support using techniques known to those skilled in the art and the antibodies can be bound directly to the solid support or though a linking moiety. Preferably the linkage is a covalent bond although other linkages are also acceptable. In one embodiment the sperm specific antibodies are linked to the solid support via an antibody linker, wherein the linker is a secondary antibody that binds to the constant region of the sperm-specific antibody. Alternatively, the linker can be an enzymatically cleavable or photolytic linker. Linkers suitable for use in accordance with the present invention are well known to those skilled in the art.

The solid support may comprise a single solid surface, or more preferably the solid support is in particulate form. In one embodiment, the individual particles of the solid support can be used to form a column for use in recovering sperm cells from a biological sample. The particles may vary in shape and can be round, rectangular or irregularly shaped, and in one embodiment the particles are magnetized. The size of the particle is important to limit shear forces during the recovery of the sperm cells. Optimally the particle size needs to be less than 4um and more preferably the particle size will range from about 10nm to about 1um and more preferably from about 50nm to about

500nm. In one embodiment the particle size ranges from about 100nm to about 300nm. Examples of paramagnetic beads include Miltenyi Biotech 50 nm dextran-coated microbeads and Micromod Nanomag-D and Nanomag-D-COOH beads.

Advantageously the use of magnetic particles allows the system of isolating sperm specific DNA to be automated. In particular, robotic arms can be used to add, remove or transfer fluids from one vessel container to another. The computer software and the mechanical hardware necessary for conducting such automation is known to those skilled in the art and has been previously described, for example see US Patent Nos. 5,366,896 and 5,128,103 the disclosures of which are expressly incorporated herein.

In accordance with one embodiment of the present invention, a device and method is provided for isolating sperm cell DNA from a sample comprising sperm cells and other cell types. In this embodiment the sperm cells are bound to magnetic particles through a sperm specific antibody wherein the antibody binds to a sperm antigen internal to the sperm plasma membrane. The method comprises the use of a robotic arm coupled to an electromagnet, wherein the robotic arm is programmed to place an electromagnet into a first compartment that contains the forensic sample, and then remove and place the electromagnet into a second compartment where the cells are lysed. In one embodiment the device further comprises a metallic pin magnetically coupled to said electromagnet. The device can be further provided with a second magnetic source (either a fixed magnet or an electromagnet) located outside the second compartment but in close enough proximity to the second compartment so as to impart a magnetic force on the contents of the second compartment. This magnet is used to assist in removing the magnetic particle from the first electromagnet after that electromagnet has been deactivated.

The device can be further provided with automated means for dispensing liquid into and withdrawing liquid from the first and second compartments. These automated dispensing and withdrawing means may comprise a system of positive and negative pressure pumps that direct fluids through tubes to the first and second compartments. Alternatively, the automated dispensing and withdrawing means may comprise one or more dispensing tubes attached to separate robotic arms wherein the dispensing and withdrawal of fluids to specific compartment is programmed.

The present invention also anticipates the use of an immunochromatographic device sensitive enough to detect trace amounts of sperm in

forensic samples. The product objective is a device termed SpermCheck. This device would be used as a first line of testing to detect the presence of sperm in forensic samples. This test device does not require a microscope evaluation and could give a yes/no answer within five minutes of applying a suspension of cells eluted from a sample. The presence of sperm would be identified by their binding to a first sperm specific antibody, wherein the first antibody is linked to a detectable label, and binding to a second sperm specific antibody, wherein the second antibody is linked to a solid surface. Chromatic means are used to move a loaded sample to a target area of the solid surface where the second antibody is located and captures the labeled sperm. The first and second antibodies are selected from those that bind to sperm antigens that are located internal to the sperm plasmid membrane. In one embodiment the target antigens are selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6.

Example 1

Protocol for Staining Sperm Containing Forensic Samples

1. Samples collected on cotton-tipped swabs are rehydrated in 0.5ml PBS per swab for 20 minutes, agitated manually at 5 minute intervals to release sample into PBS. 25 microliters of sample is applied to microscope slide and air dried at room temperature.

2. Sample on slide is rehydrated with PBS for 5 minutes, the PBS aspirated, fixed with 4% paraformaldehyde for 20 minutes, and washed two times with PBS.

3. Sample is blocked with 10% normal goat serum in PBS for 30 minutes at room temperature.

4. Blocking solution is aspirated and monoclonal antibody conjugated to fluorophore is applied to sample at a concentration of 10 micrograms per ml in PBS and incubated for 2 hours at room temperature in a humidified chamber.

5. Antibody is aspirated and the sample is washed five times with PBS.

6. Antifade reagent is applied and the sample is covered with a coverslip and sealed with nail polish. Slides are stored flat in a covered folder and stored at 4C.

We have tested the directly labeled monoclonal antibodies [single step reagent] and have very satisfactory results. Figure 1 and Figure 2 illustrate the intensity and specificity of these conjugated monoclonal antibodies. These figures represent studies where very few sperm were present in a given field and some sperm heads and tails were separated. The ability of the probe to identify head bars and tails is highlighted in these figures. AlexaFluor 488 fluorescent dye from Molecular Probes was used to label the monoclonal antibodies. It has absorption and emission wavelengths of 494nm and 519 nm respectively. This wavelength can be observed with filters commonly used to observe FITC fluorophores. The fluorophore to protein ratio of our current conjugates is 2 compared to the optimal range of 4-9. We are also examining sperm collected on swabs up to 72 hrs after intercourse. Preliminary results indicate that sperm still retain the target for these antibodies at this time point.

It is anticipated that fluorescently labeled sperm specific antibodies will be the best reagents for the purpose of unequivocally identifying sperm in forensic samples. In our tests the fluorescent signal is bright and sperm are easily distinguished from the background and other contaminating cell types. We recognize however, that until every forensic lab has use of a fluorescent microscope an alternative approach using these same specific antibodies would be of great value. For that reason the presence invention also anticpates the use of horseradish peroxidase (HRP) conjugates of the antibodies to immunostain sperm in forensic samples. HRP conjugates stain cells by causing precipitation of a colored substrate where the antibody is bound to the cell. Commercially available substrates to be tested include True Blue® (tetramethyl benzidine, TMB) from KPL Laboratories and NovaRED® from Vector Laboratories.

Example 2

Loss of Sperm Specific Antigen

Following our Human Investigation Committee approved protocol (HIC #9297) post-coital swabs were collected from 39 volunteer couples. After obtaining informed consent from a volunteer couple, they are given sample collection kits consisting of cotton swabs and labeled boxes with holes that allow the swabs to air dry. These are the same boxes used in sexual assault evidence kits in Virginia hospitals. From each of the volunteer couple, 10 vaginal swabs are collected at each of 4 time points

ranging between 1 hour and 72 hours after consensual sexual intercourse. Initially, samples were investigated at the 2, 6, 12, and 24 hour time points. In some cases swabs were collected at 1, 12, 24, and 72 hours after intercourse. Buccal swabs were also collected from male and from female partners to provide control DNA. Swabs were stored in coolers with ice blocks until brought to the study coordinator. They were then stored at 4°C with dessicant to insure uniformity and to prevent bacterial growth. The samples were then stained with the S19 antibody (described in US Patent No. 5,830,472, the disclosure of which is incorporated herein) which binds to a unique human sperm surface carbohydrate epitope, sperm agglutination antigen-1 [SAGA-1]. This antigen is synthesized in the principal cells of the epididymis, is specific to the male reproductive tract of humans and higher primates and is inserted by way of a glycophosphotidylinositol (GPI) anchor into all domains of the human sperm surface: the head and the midpiece, principal piece, and end piece of the tail.

Electron microscopy reveals loss of plasma membrane from sperm collected using current forensic techniques employing cotton swabs.

Immunofluorescent experiments indicated that the S19 mAb bound intensely to fresh ejaculated sperm but bound variably and irregularly to sperm eluted from post-coital swabs. This suggested that the SAGA-1 antigen may be lost during the collection and storage process of forensic swabs. The loss of the SAGA-1 antigen at some step in the processing might be specific to this antigen or might represent a general loss of the sperm plasma membrane. To test the latter hypothesis, we examined the fine structure of the sperm plasma membrane and the overall morphology of three groups: 1) ejaculated sperm that received no additional treatment prior to embedding for EM; 2) ejaculated sperm that were air-dried onto swabs, stored for 6 days at room temperature, and recovered prior to embedding; and 3) sperm eluted from post-coital swabs collected one or two hours after intercourse.

Results indicated that the majority of fresh sperm had intact plasma membranes as well as inner and outer acrosomal membranes. However, air-drying fresh sperm had the effect of disrupting the plasma membrane and the acrosome compartment while apparently not affecting the nuclear contents. Post-coital sperm recovered from swabs were completely stripped of plasma membrane overlying the anterior sperm head and the outer acrosomal membrane over the principal segment of the acrosome. Some

sperm eluted from swabs retained the plasma membrane overlying the equatorial segment. Thus, these results indicate that methods currently employed for the collection of sexual assault evidence using swabs may not permit the isolation of sperm using reagents targeted to the plasma membrane, such as SAGA-1. Further, it has been observed that many of the sperm eluted from swabs have been detached from their flagellum.

New sperm-specific antigens proposed as targets for sperm immunoselection from forensic samples.

The EM analysis of sperm recovered from dried swabs has led us to conclude that antigens located on the plasma membrane, such as SAGA-1 are not the best targets for sperm immunoselection. This has led us to consider other sperm proteins as possible forensic targets. Because the sperm head is often separated from the tail in sexual assault evidence recovered from swabs, one source of potential target proteins are sperm head proteins, and in one embodiment they are selected from the following:

ESP	Equatorial Segment Protein, localized to the equatorial segment of the sperm head.
SPAN-X	Major component of the cytoplasmic droplet and localized to the nuclear membrane of 50% of all sperm.
CBP86	Calcium Binding Protein 86, localized to the fibrous sheath of the principal piece of the sperm tail.
SP-10	Acrosomal matrix protein also associated with acrosomal membranes. Some SP-10 remains on the inner acrosomal membrane and in the equatorial segment after the acrosome reaction.
SAMP14	Sperm acrosomal membrane-associated protein 14, localized to the acrosome of the sperm.
SAMP32	Sperm acrosomal membrane-associated protein 32, localized to inner acrosomal membrane and the equatorial segment of the sperm head.

In addition protamine is an extremely abundant protein found only in the sperm nucleus and may prove to be an effective target on sperm heads recovered from swabs.

We are currently in the process of examining slides of swab smears collected at different time points after intercourse. The slides were prepared by pooling the cells eluted from post-coital swabs of three different couples for each time point. The following table summarizes our preliminary results to date. More time will be required to

examine a sufficient number of samples, particularly at the longer time points where few sperm are present, in order to determine the extent to which each antigen persists on sperm in the vagina after intercourse. (+) indicates positive staining observed, (-) indicates no staining observed, and (ND) indicates data not yet available.

	1 HOUR	6 HOUR	12 HOUR	24 HOUR	72 HOUR
ESP mAb 3C6	+	+	-	**ND**	**ND**
SPAN-X mAb A9	+				
CABYR Rat polyclonal	+				
SP-10 mAb MHS10	+				
SAMP32	+				
AKAP3 Rat polyclonal	+		+		

Example 3

Towards a SpermCheck Forensics Device

We are currently in clinical trials with prototypes of SpermCheck Vasectomy that has been engineered to give a positive signal in post-vasectomy samples with more than 100,000 sperm/ml. We have also produced prototypes of devices with much greater sensitivity that can detect as little as 10,000 sperm/ml. We have not yet manufactured any devices designed specifically for forensic samples but the following table summarizes the results of a very limited trial with some available prototypes designed to detect the higher sperm concentration limits of SpermCheck Vasectomy. Post-coital samples were collected on cotton swabs from three couples at 1, 6, 12, and 24 hours after intercourse. The dried swabs were stored at 4°C for more than one year. Each swab was rehydrated in 0.5 ml 10 mM phosphate, 2% Triton X-100, pH 7.2 and agitated to suspend any material extracted from the swab. For each test 140 ul of swab extract was added to the sample well of a device and the result was read after five minutes. Even these devices designed for a higher sperm concentration detection limit

were apparently able to detect sperm in samples collected one hour and 6 hours after intercourse.

Couple	1 hour	6 hour	12 hour	24 hour
#50	Strong positive	Negative	Negative	Negative
#29	Strong positive	Positive	Negative	Negative
#24	positive	Positive	Barely perceptible	Negative

These encouraging results point the way to optimizing a more sensitive device for detecting sperm in forensic samples. The more sensitive SpermCheck Forensic devices would be tested on post-coital samples collected up to 72 hours after intercourse. It will be necessary to carefully control any non-specific background reaction to insure that the devices can detect low levels of sperm without producing false positives in samples that contain no sperm. Such a five minute test device would be of great value to quickly indicate the presence of sperm without the need for a microscope and laboratory setting.

Fig 1

ESP mAb 3C6 conjugated to AlexaFluor 488 identifies sperm head in post-coital sample eluted from cotton swab.

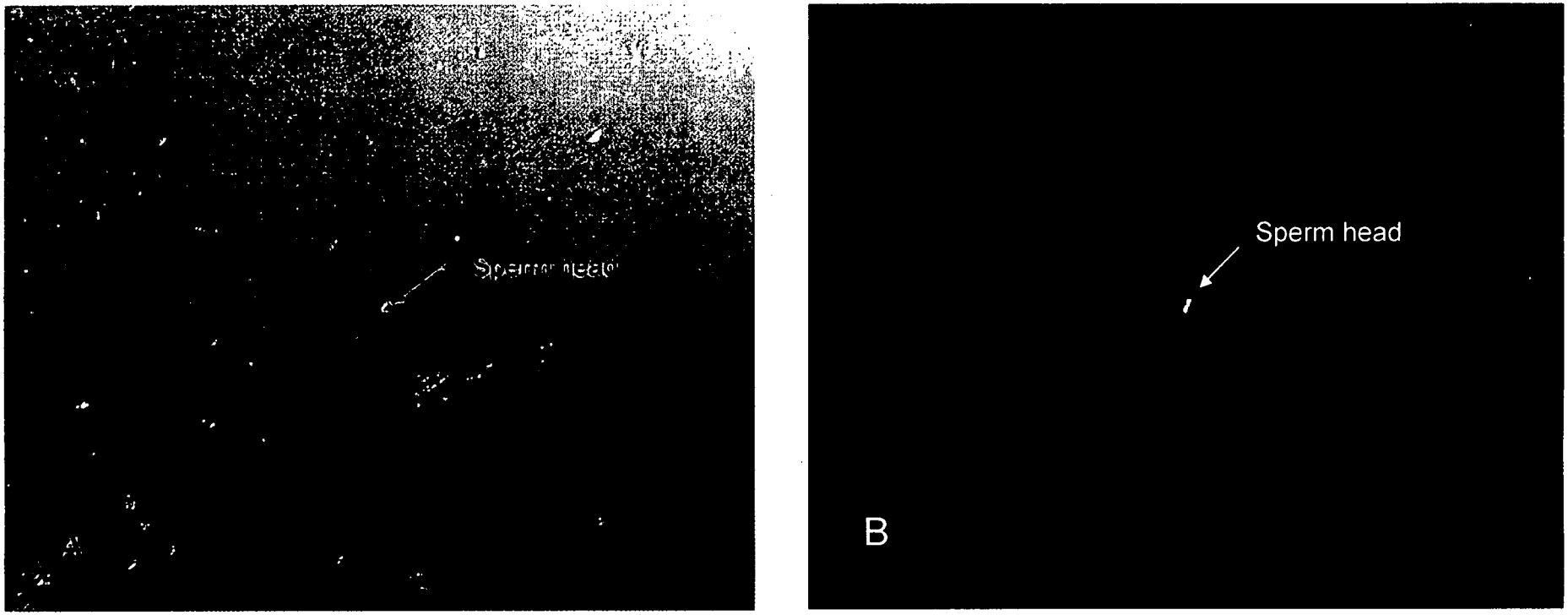

Figure 1. Phase contrast (A) and immunofluorescent image (B). Anti-ESP mAb stains the equatorial segment of a sperm eluted from a post coital sample. The equqtorial segment staining pattern is a distinctive band across the mid-region of the sperm. Other biological material in this field is not stained with the antibody so that the sperm head is clearly visible against a dark background.

The post coital sample was collected with a cotton swab one hour after intercourse, dried and stored at 4°C for approximately two years. Swabs were rehydrated with PBS. Cells and other material released from swab were spotted on microscope slides and stained with fluorescently labeled antibody according to standard protocol (see appendix).

Fig 2

CABYR-A mAb 3A4 conjugated to AlexaFluor 488 identifies sperm tails in post-coital sample eluted from a cotton swab

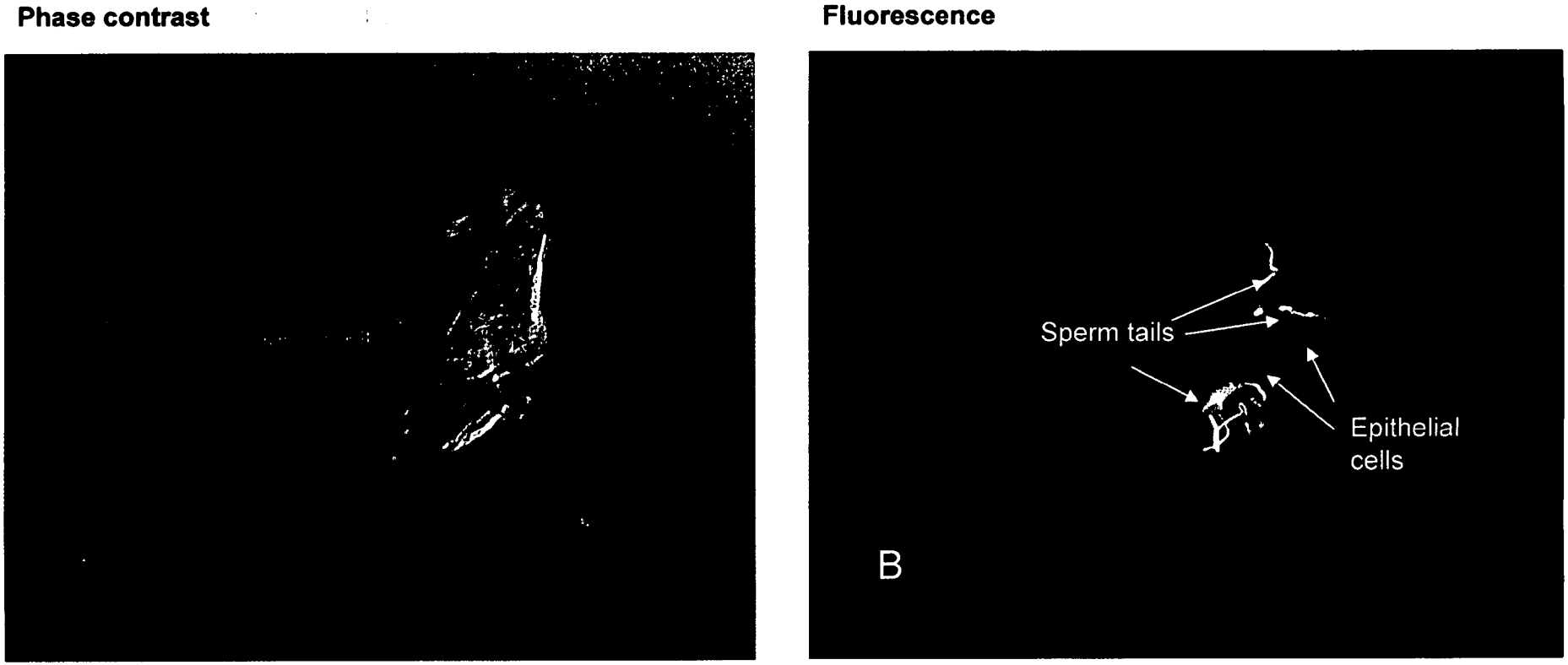

Figure 2. Phase Contrast (A) and immunofluorescent image (B). Anti-CABYR-A mAb stains the principal piece of sperm tails eluted from a post coital sample. In this field, sperm are attached to epithelial cells and are difficult to resolve in the phase contrast view at left but stand out brilliantly against a dark background with fluorescently labeled antibody on the right due to the flourescence of the sperm flagellum.

The post coital sample was collected with a cotton swab one hour after intercourse, dried and stored at 4°C for approximately two years. Swabs were rehydrated with PBS. Cells and other material released from swab were spotted on microscope slides and stained with fluorescently labeled antibody according to standard protocol (see appendix).

SEQUENCE LISTING

As used herein, the term "SP-10 antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 1 or a fragment of SEQ ID NO: 1.

SEQ ID NO: 1:. MetAsnArgPheLeuLeuLeuMetSerLeuTyrLeuLeuGlySerAla
ArgGlyThrSerSerGlnProAsnGluSerSerGlySerIleAspHis
GlnThrSerValGlnGlnLeuProGlyGluPhePheSerLeuGluAsn
ProSerAspAlaGluAlaLeuTyrGluThrSerSerGlyLeuAsnThr
LeuSerGluHisGlySerSerGluHisGlySerSerLysHisThrVal
AlaGluHisThrSerGlyGluHisAlaGluSerGluHisAlaSerGly
GluProAlaAlaThrGluHisAlaGluGlyGluHisThrValGlyGlu
GlnProSerGlyGluGlnProSerGlyGluHisLeuSerGlyGluGln
ProLeuSerGluLeuGluSerGlyGluGlnProSerAspGluGlnPro
SerGlyGluHisGlySerGlyGluGlnProSerGlyGluGlnAlaSer
GlyGluGlnProSerGlyGluHisAlaSerGlyGluHisAlaSerGly
GluGlnSerLeuGlyGluHisAlaLeuSerGluLysProSerGlyGlu
GlnAlaSerGlyAlaProIleSerSerThrSerThrGlyThrIleLeu
AsnCysTyrThrCysAlaTyrMetAsnAspGlnGlyLysCysLeuArg
GlyGluGlyThrCysIleThrGlnAsnSerGlnGlnCysMetLeuLys
LysIlePheGluGlyGlyLysLeuGlnPheMetValGlnGlyCysGlu
AsnMetCysProSerMetAsnLeuPheSerHisGlyThrArgMetGln
IleIleCysCysArgAsnGlnSerPheCysAsnLysIle

As used herein, the term "CABYR antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 2 or a fragment of SEQ ID NO: 2.

SEQ ID NO: 2:

```
Met Ile Ser Ser Lys Pro Arg Leu Val Val Pro Tyr Gly Leu Lys Thr
  1               5                  10                  15

Leu Leu Glu Gly Ile Ser Arg Ala Val Leu Lys Thr Asn Pro Ser Asn
             20                  25                  30

Ile Asn Gln Phe Ala Ala Ala Tyr Phe Gln Glu Leu Thr Met Tyr Arg
         35                  40                  45

Gly Asn Thr Thr Met Asp Ile Lys Asp Leu Val Lys Gln Phe His Gln
     50                  55                  60

Ile Lys Val Glu Lys Trp Ser Glu Gly Thr Thr Pro Gln Lys Lys Leu
```

65 70 75 80

Glu Cys Leu Lys Glu Pro Gly Lys Thr Ser Val Glu Ser Lys Val Pro
85 90 95

Thr Gln Met Glu Lys Ser Thr Asp Thr Asp Glu Asp Asn Val Thr Arg
100 105 110

Thr Glu Tyr Ser Asp Lys Thr Thr Gln Phe Pro Ser Val Tyr Ala Val
115 120 125

Pro Gly Thr Glu Gln Thr Glu Ala Val Gly Gly Leu Ser Ser Lys Pro
130 135 140

Ala Thr Pro Lys Thr Thr Thr Pro Pro Ser Ser Pro Pro Pro Thr Ala
145 150 155 160

Val Ser Pro Glu Phe Ala Tyr Val Pro Ala Asp Pro Ala Gln Leu Ala
165 170 175

Ala Gln Met Leu Gly Lys Val Ser Ser Ile His Ser Asp Gln Ser Asp
180 185 190

Val Leu Met Val Asp Val Ala Thr Ser Met Pro Val Val Ile Lys Glu
195 200 205

Val Pro Ser Ser Glu Ala Ala Glu Asp Val Met Val Ala Ala Pro Leu
210 215 220

Val Cys Ser Gly Lys Val Leu Glu Val Gln Val Val Asn Gln Thr Ser
225 230 235 240

Val His Val Asp Leu Gly Ser Gln Pro Lys Glu Asn Glu Ala Glu Pro
245 250 255

Ser Thr Ala Ser Ser Val Pro Leu Gln Asp Glu Gln Glu Pro Pro Ala
260 265 270

Tyr Asp Gln Ala Pro Glu Val Thr Leu Gln Ala Asp Ile Glu Val Met
275 280 285

Ser Thr Val His Ile Ser Ser Val Tyr Asn Asp Val Pro Val Thr Glu
290 295 300

Gly Val Val Tyr Ile Glu Gln Leu Pro Glu Gln Ile Val Ile Pro Phe
305 310 315 320

Thr Asp Gln Val Ala Cys Leu Lys Glu Asn Glu Gln Ser Lys Glu Asn
325 330 335

Glu Gln Ser Pro Arg Val Ser Pro Lys Ser Val Val Glu Lys Thr Thr
340 345 350

Ser Gly Met Ser Lys Lys Ser Val Glu Ser Val Lys Leu Ala Gln Leu
355 360 365

Glu Glu Asn Ala Lys Tyr Ser Ser Val Tyr Met Glu Ala Glu Ala Thr
370 375 380

Ala Leu Leu Ser Asp Thr Ser Leu Lys Gly Gln Pro Glu Val Pro Ala
385 390 395 400

Gln Leu Leu Asp Ala Glu Gly Ala Ile Lys Ile Gly Ser Glu Lys Ser
405 410 415

Leu His Leu Glu Val Glu Val Thr Ser Ile Val Ser Asp Asn Thr Gly
420 425 430

Gln Glu Glu Ser Gly Glu Asn Ser Val Pro Gln Glu Met Glu Gly Arg
435 440 445

Pro Val Leu Ser Gly Glu Ala Ala Glu Ala Val His Ser Gly Thr Ser
450 455 460

Val Lys Ser Ser Ser Gly Pro Phe Pro Pro Ala Pro Glu Gly Leu Thr
465 470 475 480

Ala Pro Glu Ile Glu Pro Glu Gly Glu Ser Thr Ala Glu Gly Leu Met
485 490 495

Lys Pro Ala Met Ala Thr Ser Glu Arg Gly Gln Pro Pro Pro Cys Ser
500 505 510

Asn Met Trp Thr Leu Tyr Cys Leu Thr Asp Lys Asn Gln Gln Gly His

515 520 525

Pro Ser Pro Pro Pro Ala Pro Gly Pro Phe Pro Gln Ala Thr Leu Tyr
530 535 540

Leu Pro Asn Pro Lys Asp Pro Gln Phe Gln Gln His Pro Pro Lys Val
545 550 555 560

Thr Phe Pro Thr Tyr Val Met Gly Asp Thr Lys Lys Thr Ser Ala Pro
565 570 575

Pro Phe Ile Leu Val Gly Ser Asn Val Gln Glu Ala Gln Gly Trp Lys
580 585 590

Pro Leu Pro Gly His Ala Val Val Ser Gln Ser Asp Val Leu Arg Tyr
595 600 605

Val Ala Met Gln Val Pro Ile Ala Val Pro Ala Asp Glu Lys Tyr Gln
610 615 620

Lys His Thr Leu Ser Pro Gln Asn Ala Asn Pro Pro Ser Gly Gln Asp
625 630 635 640

Val Pro Arg Pro Lys Ser Pro Val Phe Leu Ser Val Ala Phe Pro Val
645 650 655

Glu Asp Val Ala Lys Lys Ser Ser Asp Ser Gly Asp Lys Cys Ala Pro
660 665 670

Phe Gly Ser Tyr Gly Ile Ala Gly Glu Val Thr Val Thr Thr Ala His
675 680 685

Lys Arg Arg Lys Ala Glu Thr Glu Asn
690 695

As used herein, the term "ESP antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 3 or a fragment of SEQ ID NO: 3.

SEQ ID NO: 3:

```
Met Lys Pro Leu Val Leu Leu Val Ala Leu Leu Leu Trp Pro Ser Ser
  1               5                  10                  15

Val Pro Ala Tyr Pro Ser Ile Thr Val Thr Pro Asp Glu Glu Gln Asn
             20                  25                  30

Leu Asn His Tyr Ile Gln Val Leu Glu Asn Leu Val Arg Ser Val Pro
         35                  40                  45

Ser Gly Glu Pro Gly Arg Glu Lys Lys Ser Asn Ser Pro Lys His Val
     50                  55                  60

Tyr Ser Ile Ala Ser Lys Gly Ser Lys Phe Lys Glu Leu Val Thr His
 65                  70                  75                  80

Gly Asp Ala Ser Thr Glu Asn Asp Val Leu Thr Asn Pro Ile Ser Glu
                 85                  90                  95

Glu Thr Thr Thr Phe Pro Thr Gly Gly Phe Thr Pro Glu Ile Gly Lys
            100                 105                 110

Lys Lys His Thr Glu Ser Thr Pro Phe Trp Ser Ile Lys Pro Asn Asn
        115                 120                 125

Val Ser Ile Val Leu His Ala Glu Glu Pro Tyr Ile Glu Asn Glu Glu
    130                 135                 140

Pro Glu Pro Glu Pro Glu Pro Ala Ala Lys Gln Thr Glu Ala Pro Arg
145                 150                 155                 160

Met Leu Pro Val Val Thr Glu Ser Ser Thr Ser Pro Tyr Val Thr Ser
                165                 170                 175
```

```
Tyr Lys Ser Pro Val Thr Thr Leu Asp Lys Ser Thr Gly Ile Glu Ile
            180                 185                 190

Tyr Thr Glu Ser Glu Asp Val Pro Gln Leu Ser Gly Glu Thr Ala Ile
        195                 200                 205

Glu Lys Pro Glu Glu Phe Gly Lys His Pro Glu Ser Trp Asn Asn Asp
    210                 215                 220

Asp Ile Leu Lys Lys Ile Leu Asp Ile Asn Ser Gln Val Gln Gln Ala
225                 230                 235                 240

Leu Leu Ser Asp Thr Ser Asn Pro Ala Tyr Arg Glu Asp Ile Glu Ala
                245                 250                 255

Ser Lys Asp His Leu Lys Pro Ser Leu Ala Leu Ala Ala Ala Ala Glu
            260                 265                 270

His Lys Leu Lys Thr Met Tyr Lys Ser Gln Leu Leu Pro Val Gly Arg
        275                 280                 285

Thr Ser Asn Lys Ile Asp Asp Ile Val Thr Val Ile Asn Met Leu Cys
    290                 295                 300

Asn Ser Arg Ser Lys Leu Tyr Glu Tyr Leu Asp Ile Lys Cys Val Pro
305                 310                 315                 320

Pro Glu Met Arg Glu Lys Ala Ala Thr Val Phe Asn Thr Leu Lys Asn
                325                 330                 335

Met Cys Arg Ser Arg Arg Val Thr Ala Leu Leu Lys Val Tyr
            340                 345                 35
```

As used herein, the term "SAMP32 antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 4 or a fragment of SEQ ID NO: 4.

SEQ ID NO: 4:

```
Met Ser Pro Arg Gly Thr Gly Cys Ser Ala Gly Leu Leu Met Thr Val
 1               5                  10                  15

Gly Trp Leu Leu Leu Ala Gly Leu Gln Ser Ala Arg Gly Thr Asn Val
            20                  25                  30

Thr Ala Ala Val Gln Asp Ala Gly Leu Ala His Glu Gly Glu Gly Glu
        35                  40                  45

Glu Glu Thr Glu Asn Asn Asp Ser Glu Thr Ala Glu Asn Tyr Ala Pro
    50                  55                  60

Pro Glu Thr Glu Asp Val Ser Asn Arg Asn Val Val Lys Glu Val Glu
65                  70                  75                  80

Phe Gly Met Cys Thr Val Thr Cys Gly Ile Gly Val Arg Glu Val Ile
                85                  90                  95

Leu Thr Asn Gly Cys Pro Gly Gly Glu Ser Lys Cys Val Val Arg Val
            100                 105                 110

Glu Glu Cys Arg Gly Pro Thr Asp Cys Gly Trp Gly Lys Pro Ile Ser
        115                 120                 125

Glu Ser Leu Glu Ser Val Arg Leu Ala Cys Ile His Thr Ser Pro Leu
    130                 135                 140

Asn Arg Phe Lys Tyr Met Trp Lys Leu Leu Arg Gln Asp Gln Gln Ser
145                 150                 155                 160

Ile Ile Leu Val Asn Asp Ser Ala Ile Leu Glu Val Arg Lys Glu Ser
                165                 170                 175

His Pro Leu Ala Phe Glu Cys Asp Thr Leu Asp Asn Asn Glu Ile Val
            180                 185                 190
```

```
Ala Thr Ile Lys Phe Thr Val Tyr Thr Ser Ser Glu Leu Gln Met Arg
        195                 200                 205

Arg Ser Ser Leu Pro Ala Thr Asp Ala Ala Leu Ile Phe Val Leu Thr
    210                 215                 220

Ile Gly Val Ile Ile Cys Val Phe Ile Ile Phe Leu Leu Ile Phe Ile
225                 230                 235                 240

Ile Ile Asn Trp Ala Ala Val Lys Ala Phe Trp Gly Ala Lys Ala Ser
                245                 250                 255

Thr Pro Glu Val Gln Ser Glu Gln Ser Ser Val Arg Tyr Lys Asp Ser
            260                 265                 270

Thr Ser Leu Asp Gln Leu Pro Thr Glu Met Pro Gly Glu Asp Asp Ala
        275                 280                 285

Leu Ser Glu Trp Asn Glu
    290
```

As used herein, the term "SPAN-X antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 5 or a fragment of SEQ ID NO: 5.

SEQ ID NO: 5: International Application PCT/US99/24973, the disclosure of which is incorporated herein

As used herein, the term "AKAP antibody" and like terms refers to an antibody that specifically binds to a polypeptide comprising SEQ ID NO: 6 or a fragment of SEQ ID NO: 6 as provided by GenBank accession number AF087003.